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Predictive Thermal Inactivation Model for Effects of Temperature, Sodium Lactate, NaCl, and Sodium Pyrophosphate on *Salmonella* Serotypes in Ground Beef

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Analyses of survival data of a mixture of *Salmonella* spp. at fixed temperatures between 55°C (131°F) and 71.1°C (160°F) in ground beef matrices containing concentrations of salt between 0 and 4.5%, concentrations of sodium pyrophosphate (SPP) between 0 and 0.5%, and concentrations of sodium lactate (NaL) between 0 and 4.5% indicated that heat resistance of *Salmonella* increases with increasing levels of SPP and salt, except that, for salt, for larger lethalities close to 6.5, the effect of salt was evident only at low temperatures (<64°C). NaL did not seem to affect the heat resistance of *Salmonella* as much as the effects induced by the other variables studied. An omnibus model for predicting the lethality for given times and temperatures for ground beef matrices within the range studied was developed that reflects the convex survival curves that were observed. However, the standard errors of the predicted lethalities from this models are large, so consequently, a model, specific for predicting the times needed to obtained a lethality of 6.5 $\log_{10}$, was developed, using estimated results of times derived from the individual survival curves. For the latter model, the coefficient of variation (CV) of predicted times range from about 6 to 25%. For example, at 60°C, when increasing the concentration of salt from 0 to 4.5%, and assuming that the concentration of SPP is 0%, the time to reach a 6.5-$\log_{10}$ relative reduction is predicted to increase from 20 min (CV = 11%) to 48 min (CV = 15%), a 2.4 factor (CV = 19%). At 71.1°C (160°F) the model predicts that more than 0.5 min is needed to achieve a 6.5-$\log_{10}$ relative reduction.

Salmonella enterica has long been recognized as an important food-borne pathogen, and it continues to be a leading cause of food-borne disease outbreaks associated with consumption of meat and poultry. The annual incidence of salmonellosis in the US is 1.4 million cases, causing as many as 550 deaths (12), and the incidence of salmonellosis has increased during the past 30 years. This reality stems from the ubiquitous occurrence of *Salmonella* in the environment, its prominence in various sectors of the agriculture industry, and the escalating movement of food and food ingredients worldwide. Important contributing factors which lead to outbreaks of food-borne illness, including salmonellosis, are inadequate time and/or temperature exposure during initial thermal processing (or cooking) and inadequate reheating to kill pathogens in retail food service establishments or homes (2, 15). Inadequate cooking was cited as a contributing factor in 67% of the outbreaks in which *Salmonella* was an etiological agent (2). These outbreaks have implicated a variety of foods, including meat and poultry, milk, ice cream, cheese, eggs and egg products, chocolate, and spices, as vehicles of transmission (4). Current performance standards require that thermal processing schedules must achieve a 6.5-$\log_{10}$ reduction of *Salmonella* for cooked beef, ready-to-eat roast beef, and cooked corned beef products (17).

An effective thermal process is necessary to control the potential hazard of *Salmonella* in cooked meat products. A key to optimization of the heating step is defining the target pathogen's heat resistance. While overestimating the heat resistance negatively impacts on product quality, underestimating increases the likelihood that the contaminating pathogen persists after heat treatment or cooking. Accordingly, teams of investigators have conducted thermal inactivation studies of different *Salmonella* serotypes in aqueous media and foods (4). Various factors affecting the heat resistance have been documented, including growth temperature, stage of growth, initial population, bacterial strains, composition and pH of the heating menstruum, heat shock, and methodology used for detection of survivors (16). In a study by Juneja et al. (7), when the heat resistance of *Salmonella* serotypes was quantified in beef of different fat levels, asymptotic *D* values (*D* values for large times) increased with increasing fat levels. While the study by Juneja et al. (7) provided some characterization of the inactivation kinetics, there is a lack of information on the effects of increasing concentrations of sodium pyrophosphate (SPP) and sodium lactate (NaL) in combination with various salt levels on the heat resistance of the organism. Accordingly, the present study was carried out to quantitatively assess the relative effects and interactions of SPP, NaL, NaCl, and temperature on the inactivation kinetics of *Salmonella* serotypes.

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TABLE 1. *Salmonella* serotype cocktail sources

Cocktail strains	Strain designation	Source[a]	Isolate
Salmonella serovar Thompson	FSIS 120	FSIS	Chicken
Salmonella serovar Enteritidis phage type 13A	H3527	CDC	Clinical
Salmonella serovar Enteritidis phage type 4	H3502	CDC	Clinical
Salmonella serovar Typhimurium phage type DT 104	H3380	CDC	Clinical
Salmonella serovar Hadar	MF 60404	FSIS	Turkey
Salmonella serovar Copenhagen	8457	FSIS	Pork
Salmonella serovar Montevideo	FSIS 051	FSIS	Beef
Salmonella serovar Heildelberg	F5038BG1	CDC	Environmental

[a] Abbreviations: FSIS, Food Safety Inspection Service of the U.S. Department of Agriculture; CDC, Centers for Disease Control and Prevention.

MATERIALS AND METHODS

Organisms. A cocktail consisting of eight strains of different serotypes of *Salmonella* representing isolates from beef, pork, chicken, or turkey or from human clinical cases was used in this study. The information about these strains is given in Table 1. These strains were preserved by freezing the cultures at −70°C in vials containing tryptic soy broth (Difco laboratories, Inc., Detroit, Mich.) supplemented with 10% (vol/vol) glycerol (Sigma Chemical Co., St. Louis, Mo.).

Products. Raw 75% lean ground beef, used as the heating menstruum, was obtained from a retail supermarket. The meat was separated into batches for different treatments and mixed thoroughly with the additives to be tested, i.e., each batch received various variable combinations of SPP (0.0 to 0.3%, wt/wt), NaL (0.0 to 4.8%, wt/wt), and/or NaCl (0.0 to 4.5%, wt/wt). The pH of the meats tested were determined using a combination electrode (Sensorex, semimicro; A.H. Thomas, Philadelphia, Pa.) attached to a pH meter (model 310; Orion, Boston, Mass.). The meat was placed into stomacher 400 polyethylene bags (50 g/bag) and vacuum sealed. Thereafter, five of these bags were vacuum sealed in barrier pouches (Bell Fibre Products, Columbus, Ga.), frozen at −40°C, and irradiated (42 kGy) to eliminate indigenous microflora. Random samples were tested to verify elimination or inactivation of microflora by diluting in 0.1% (wt/vol) peptone water (PW) to obtain1:1 meat slurry, followed by direct surface plating the suspension (0.1 and 1.0 ml) on tryptic soy agar (TSA) (Difco) and incubating aerobically at 30°C for 48 h.

Preparation of test cultures. To propagate the cultures, vials were partially thawed at room temperature and 1.0 ml of the thawed culture was transferred to 10 ml of brain heart infusion (BHI) (Difco) broth in 50-ml tubes and incubated for 24 h at 37°C. This culture was not used in heating tests, due to the presence of freeze-damaged cells. The inocula for use in heating tests were prepared by transferring 0.1 ml of each culture to tubes of BHI broth (10 ml) and incubating aerobically for 24 h at 37°C. These cultures were then maintained in BHI for 2 weeks at 4°C. A new series of cultures was initiated from the frozen stock on a biweekly basis.

A day before the experiment, the inocula for conducting the heating studies were prepared by transferring 0.1 ml of each culture to 50 ml of BHI in 250-ml flasks, and incubating aerobically for 18 h at 37°C to provide late-stationary-phase cells. On the day of the experiment, each culture was centrifuged (5,000 × *g*, 15 min, 4°C), the pellet was washed twice in 0.1% (wt/vol) PW and finally suspended in PW to a target level of 8 to 9 $\log_{10}$ CFU/ml. The population densities in each cell suspension were enumerated by spiral plating (model D; Spiral Biotech, Bethesda, Md.) appropriate dilutions (in 0.1% PW), in duplicate, onto TSA plates. Approximately equal volumes of each culture were combined in a sterile conical vial to obtain an eight-strain mixture of *Salmonella* (8 $\log_{10}$ CFU/ml) prior to inoculation of meat.

Experimental design. A fractional factorial design was used to assess the effects and interactions of heating temperature, SPP, NaL, and NaCl. Levels of the factors studied are as follows: heating temperature, 55, 60, 65, and 71.1°C; NaCl, 0.0, 0.75, 1.5, 2.5, 3.0, 3.75, and 4.5%; SPP, 0.0, 0.15, 0.30, 0.40, 0.45, and 0.50%; NaL, 0.0, 1.0, 1.5, 2.5, 3.0, 4.0, and 4.5%.

Forty-five different design points of the above factors were studied. Table 2 gives the 45 design points tested along with some other information as explained below. For each experimental combination at least two replicates were obtained, and in total there were 110 survivor curves, two per experimental combination, for a total of 55 combinations, some of these the same, to give 45 distinct combinations.

Sample preparation and inoculation. The cocktail of eight strains of *Salmonella* was added (0.1 ml) to 50 g of thawed, irradiated ground meat. The inoculated meat was blended with a Seward laboratory stomacher 400 for 5 min to ensure even distribution of the organisms in the meat sample. Duplicate 3-g ground-meat samples were then weighed aseptically into sterile filtered stomacher bags (30 by 19 cm; Spiral Biotech). Negative controls consisted of bags containing meat samples inoculated with 0.1 ml of 0.1% (wt/vol) PW with no bacterial cells. Thereafter, the bags were compressed into a thin layer (approximately 0.5 to 1 mm thick) by pressing them against a flat surface, excluding most of the air, and then were heat sealed using a Multivac (model A300/16; Multivac Inc., Kansas City, Mo.) packaging machine.

Thermal inactivation and bacterial enumeration. The thermal inactivation studies were carried out in a temperature controlled circulating water bath (Techne, ESRB, Cambridge, United Kingdom) stabilized at 55, 60, 65, or 71.1°C according to the procedure as described by Juneja et al. (8). Bags for each replicate were then removed at predetermined time intervals, placed into an ice-water bath and analyzed within 30 min. Surviving bacteria were enumerated by surface plating appropriate dilutions, in duplicate, on to TSA supplemented with 0.6% yeast extract and 1% sodium pyruvate, using a spiral plater.

Samples not inoculated with *Salmonella* cocktail were plated as controls. Also, 0.1- and 1.0-ml aliquots of undiluted suspension were surface plated, where necessary. All plates were incubated at 30°C for at least 48 h prior to counting colonies. For each replicate experiment, average numbers of CFU per gram of four platings of each sampling point were used to determine estimates of the lethality kinetics.

Statistical methods. (i) Primary model. Graphical examination of the observed survival curves revealed that almost all the curves had a convex shape. Some of the curves also displayed "shoulders," suggesting a possible lag effect. The dependent variable used in the regressions is the observed $\log_{10}$ of $N(t)/N(0)$, where $N(t)$ is the number of cells at time t. The negative of this quantity is referred to as the lethality at time t. The following equation,

$$E\{\log_{10}[N(t)/N(0)]\} = -\log_{10}\{1 + \exp[a + b\ln(t)]\} \quad (1)$$

where E is the expected value operator and a and b (>0) are constants, has been used for fitting survival curves with the above described properties by various researchers (1, 10). This function provides the flexibility to fit a variety of survival curves that have asymptotic convex behavior. As t approaches infinity the derivative of the right side of equation 1 approaches 0. To allow for the possibility that, asymptotically, the survival curves approach a straight line with nonzero slope, we considered a model that involved adding another term to the exponent:

$$E\{\log_{10}[N(t)/N(0)]\} = -\log\{1 + \exp[a + b\ln(t) + ct]\} \quad (2)$$

where c is ≥ 0. The asymptotic D value for survival curve of equation 2 thus is $\ln(10)/c$. The derivative of the right side of equation 2 approaches $-e^a bt^{b-1}$, as t approaches 0 from the right, so that, if b is >1, then the slope at zero is zero, and if b is <1 then the limiting slope is minus infinity. When b is >1, the survival curve has a "shoulder" and the point (time) of inflection (where the curve becomes convex) is $[(b-1)/e^a]^{1/b}$. Thus, for a given value of b of >1 (and c), smaller values of a provide curves with more pronounced shoulders and larger points of inflections.

(ii) Secondary model. An omnibus model for predicting survival curves for any specified values of temperature, salt, SPP, and NaL, was determined by considering the parameters that are identified in equations 1 or 2 to be at most quadratic polynomials of the independent variables described in the Results section. Using higher order polynomials might result in a response surface with more than one local maximum or minimum, which would be contrary to our a priori expectations, and, given the number of design points, a result contrary to this expectation probably could not be supported and thus would not be believed but rather assumed to be a consequence of experimental error. The desire is to

TABLE 2. Estimated natural logarithm of time needed to obtain 6.5 lethality[a]

Des	Temp (°C)	NaCl (%)	SPP (%)	NaL (%)	No. of estimates	Geometric mean of estimated time (min) for 6.5 lethality	mean ln (time) for 6.5 lethality	Between-experiment SD of ln (time)	Pooled SD due to regression
1	55.0	0.00	0.00	0.0	6	116.93	4.762	0.300	0.340
2	55.0	0.00	0.00	4.5	2	171.49	5.145	0.035	0.325
3	55.0	0.00	0.50	0.0	2	237.09	5.468	0.498	0.548
4	55.0	0.00	0.50	4.5	2	336.95	5.820	0.148	0.326
5	55.0	2.50	0.30	2.5	2	255.14	5.542	0.305	0.107
6	55.0	4.50	0.00	0.0	2	1,113.4	7.015	0.179	0.321
7	55.0	4.50	0.00	4.5	2	501.33	6.217	0.009	0.240
8	55.0	4.50	0.50	0.0	2	1803.4	7.497	0.209	0.345
9	55.0	4.50	0.50	4.5	2	913.41	6.817	1.498	0.327
10	60.0	0.00	0.00	0.0	4	10.68	2.368	0.448	0.491
11	60.0	1.50	0.15	1.5	2	69.01	4.234	0.566	0.470
12	60.0	1.50	0.15	3.0	2	56.22	4.029	0.246	0.451
13	60.0	1.50	0.40	1.5	2	71.72	4.273	0.449	0.576
14	60.0	1.50	0.40	3.0	2	66.98	4.204	0.246	0.319
15	60.0	2.50	0.30	2.5	2	31.67	3.455	0.256	0.404
16	60.0	3.00	0.15	1.5	2	27.14	3.301	0.148	0.190
17	60.0	3.00	0.15	3.0	2	28.49	3.350	0.165	0.146
18	60.0	3.00	0.40	1.5	1	36.02	3.584		0.156
19	60.0	3.00	0.40	3.0	2	43.35	3.769	0.028	0.270
20	65.0	0.00	0.00	0.0	6	3.41	1.226	0.920	0.546
21	65.0	0.00	0.30	2.5	1	7.43	2.006		0.924
22	65.0	0.75	0.45	4.0	2	4.88	1.585	0.104	0.067
23	65.0	1.50	0.30	2.5	2	5.45	1.695	1.118	0.412
24	65.0	2.50	0.00	2.5	2	4.27	1.452	1.648	0.406
25	65.0	2.50	0.15	2.5	2	6.29	1.839	1.660	0.642
26	65.0	2.50	0.30	0.0	2	1.60	0.469	0.594	0.458
27	65.0	2.50	0.30	1.5	2	5.57	1.718	0.687	0.605
28	65.0	2.50	0.30	2.5	9	5.27	1.663	0.846	0.385
29	65.0	2.50	0.30	3.0	2	10.07	2.309	0.057	0.503
30	65.0	2.50	0.30	4.5	2	5.91	1.777	0.228	0.348
31	65.0	2.50	0.40	2.5	2	7.11	1.962	0.197	0.771
32	65.0	2.50	0.50	2.5	2	7.95	2.074	0.515	0.422
33	65.0	3.00	0.30	2.5	2	5.28	1.663	0.082	0.193
34	65.0	3.75	0.45	1.0	2	8.40	2.128	0.181	0.095
35	65.0	4.50	0.30	2.5	2	4.48	1.500	0.147	0.129
36	71.1	0.00	0.00	0.0	4	0.69	−0.377	0.504	0.247
37	71.1	1.50	0.15	1.5	2	0.55	−0.602	0.270	0.597
38	71.1	1.50	0.15	3.0	2	0.76	−0.271	0.325	0.612
39	71.1	1.50	0.40	1.5	2	0.72	−0.328	1.083	0.356
40	71.1	1.50	0.40	3.0	2	0.46	−0.775	0.047	0.195
41	71.1	2.50	0.00	2.5	2	0.40	−0.928	0.203	0.314
42	71.1	3.00	0.15	1.5	2	0.62	−0.479	0.006	0.125
43	71.1	3.00	0.15	3.0	2	0.47	−0.758	0.198	0.148
44	71.1	3.00	0.40	1.5	2	0.85	−0.167	0.690	0.224
45	71.1	3.00	0.40	3.0	2	1.12	0.115	0.620	0.362
Pooled SD								0.641	0.401

[a] Derived from nonlinear regressions (equation 1) for 45 combinations of temperature, salt, SPP, and NaL.

determine a model that includes only statistically significant terms since including insignificant terms increases the standard error of predictions possibly without any corresponding reduction of bias (an example of Occam's maxim). Thus, the selection of terms in a model does not preclude other variables that are not included in the model from being important for predicting lethality. Initially, stepwise regressions were used to identify statistically significant variables from a quadratic response surface for inclusion in the model. The natural logarithm of the temperature was included among the variables considered in the regression. Influential observations were determined by examining studentized residuals (computed excluding the observation) and Cook's D statistic.

One advantage of equation 1 is that the logit transformation on the quantity $1 - r(t)$, where $r(t) = N(t)/N$ (0), or equivalently, the transformation,

$$f(x) = \ln(10^{-x} - 1) \quad (3)$$

where $x = \log_{10}(rt)$, is linear in the unknown parameters, a, b, and c, so that linear mixed effects model can be used for estimating the model parameters as linear functions of the variables studied. Using linear mixed effects models accounts, in a simple way, for the correlations that exist among the observations. Using the variables identified from the stepwise regressions, a mixed effects model was fit (14). Nested error structures were assumed, where experimental condition, s, and replicate within experimental condition, $e(s)$, were considered as random effects. That is to say, if f(x) is assumed to be a linear combination of $a + b\ln(t)$, then, for example, it can be assumed that a is actually a random variable that can be expressed as: $a = \mu + \varepsilon_s + \varepsilon_{e(s)} + \varepsilon_r$, where μ is the expected value of a, ε_s is the error associated with factor s; $\varepsilon_{e(s)}$ is the error associated with the factor e nested within s; ε_r is a residual error (nested within s and e); and the error terms are independent, have zero expected values and specified variances. The same type of assumption is made for b, so that, in addition to the variances, there are possible nonzero covariances between corresponding errors at the same structural level associated with a and b. The expected value of a and b, themselves, are

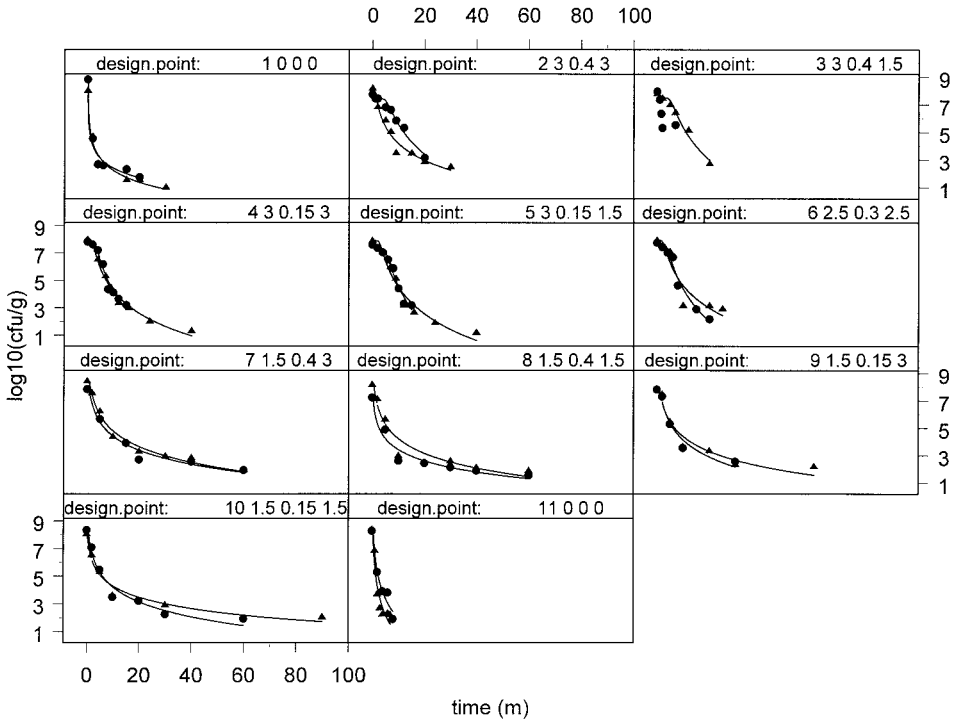

FIG. 1. Observed and fitted survival curves for the different combinations of values of salt, SPP, and NaL, for temperature = 60°C. The first number in the heading for the graphs is a design number designator; the following three values represent the salt, SPP, and NaL, values, respectively. For each design point there were two experiments; data points labeled with the same symbol are from the same experiment.

assumed to be linear combinations of the independent variables with unknown coefficients that can be assumed to be random variables. When including all possible variances and covariances, such mixed effect models can have an enormous number of parameters for which convergent solutions with estimable variances (nonsingular Hessian matrix) sometimes are not readily attainable. Consequently simplifying assumptions are made in order to reduce the number of parameters to "manageable" levels. In this case, it is assumed that only the constant or intercept terms—ones that are not coefficients of an independent variable of temperature, salt, SPP or NaL—are associated with random variables in the sense described above. For details of using these models, the book by Pinheiro and Bates (14) can be consulted; the approach given in that book was followed here. In this study, design combinations, and the replicates within these are considered as factors. In considering whether to include terms in the model, likelihood ratio tests based on the statistic $L = -2\ln(\text{likelihood ratio})$, compared to the 95th percentile of a chi-square distribution (0.05 significance level) with appropriate degrees of freedom, was used. That is, evaluating whether the addition of q terms improves the goodness-of-fit was made by comparing the difference of the statistics, -2 ln(likelihood), that are given in the PROC MIXED output, with the 95th percentile of a chi-square distribution with q degrees of freedom. With each model considered, the plots of the residuals versus the predicted values were examined. Predictions of $x = \log_{10}[r(t)]$, as a function of the selected independent variables, were obtained by using the inverse of the function of equation 3, and the standard errors of these predicted values were obtained using the linear approximation (first term of the Taylor series) of the inverse function, and the asymptotic covariance matrix of the estimated values of the parameters.

Of particular importance is the times needed to obtain a 6.5-$\log_{10}$ relative reduction. The above model could be used for estimating these times, however, a more direct approach was used: for each individual experiment, using the estimated survival curve, an estimate of the time for a predicted 6.5-$\log_{10}$ relative reduction, $t_{6.5}$, was derived, and the natural logarithm of this estimated time was used as the dependent variable in a mixed effects regression analysis, as described above. From equation 1, the predicted time, $t_{6.5}$, to obtain a 6.5 lethality is obtained as follows:

$$t_{6.5} = \exp\left[\frac{f(-6.5) - a}{b}\right] \quad (4)$$

where f is given in equation 3. (If equation 2 were used, then direct numerical procedures would be needed to solve for $t_{6.5}$.)

Nonlinear regressions, stepwise regressions, and linear mixed effects models were computed using PC SAS, release 8, using the available default options, with the exception for the mixed effects models, where the maximum-likelihood method was used.

RESULTS

Preliminary analysis. Equation 2 was fit for each growth curve, with the restriction that b be >0 and c be ≥ 0, where it was also assumed that $N(0)$ was a parameter with an unknown value. Of the 98 estimated curves for which the estimate of b was >0, 26 of them had a c of >0, and of these 6 had estimated value of c significantly greater than zero at the one-sided 0.10 level and only 2 at a significance better than 0.05. The pooled root mean square error (RMSE) for fitting equation 2 is 0.548 compared to 0.500 for equation 1. Thus, it appears that, for individual survival curves, equation 2 does not generally provide a significantly better fit than does equation 1. Hence, for this analysis, equation 1 is used.

Furthermore there were 18 values for which nondetection was recorded. For these, when it was assumed that there was 1 cell so that the $\log_{10}$ value would equal 0, using equation 1, the average predicted $\log_{10}$ value was 0.37 and only 3 of the 18 data values had positive residuals. The measurements at these levels are relatively inaccurate, and the pooled RMSE decreased slightly when not including them. The differences in the models and predictions discussed below between including these 18 values and assuming a $\log_{10}$ value of 0, and deleting these 18 values are small. For example, the model presented in this

paper (deleting the 18 results) predicts that, at 71.1°C and with salt, SPP, and NaL = 0%, the time needed to obtain a 6.5 lethality is 0.60 min with an error CV of 18.3%, while when the 18 data points are included, the estimated time is 0.54 min with CV of 19.6%; thus, the difference is about 10% lower when including the points. For 60°C for the same circumstances, there is a 5% difference: without the 18 data points, the estimated time is 20.1 min with a CV of 10.8%; with the data points, the estimate is 19.1 min with a CV of 11.5%. Insofar as the low levels associated with these 18 samples are not measured accurately; including them increases the standard error of predictions; and the model structure and basic conclusions of this paper are not affected whether or not they are included, it was decided not to include these data. With these points deleted, an examination of the residuals of the regressions using equation 1 revealed that for smallest positive times (3 s), the predicted model underestimated, on the average, the observed lethalities. The possibility exists that these values could be affected by the temperature come-up times more substantially than other values, though it is considered that the come-up time is negligible. Consequentially, data for times equal 3 seconds were deleted.

Figures 1 and 2 contains plots of the observed data and the fitted curves for 60 and 71.1°C, respectively. The headings include the order number of the design point, followed by the concentrations of salt, SPP, and NaL. For each design point, there were two replicate experiments; in the figures, the data points labeled by the same symbol are from the same experiment. These graphs show the fit of equation 1 to the observed data; similar patterns exist for the other temperatures. Figures showing the observed data and fitted curves derived from the omnibus model are given later. For the 110 fitted survival curves using equation 1, the pooled RMSE was 0.480 $\log_{10}$, and the average *R*-square values was 0.971; however, there was a fitted survival curve that had an exceptionally low *R*-square value of 0.75 and which had only five measured values where the difference between the lowest and highest values was 2.64 $\log_{10}$. In the appendix is a table that gives the estimated parameter values of *a* and *b*, and the estimated times to obtain a 6.5 lethality for each survival curve.

Stepwise regressions of the estimated parameters, *a* and *b*, and the estimated natural logarithm of the times needed to obtain a 6.5 $\log_{10}$ lethality, $\ln(t_{6.5})$, obtained from equation 4, were performed, where the independent variables consisted of all possible terms of a quadratic polynomial in temperature, ln(temperature) salt, SPP, and NaL. Two observations were found to have large studentized residuals (greater than 3 in absolute value) for predicting $\ln(t_{6.5})$. These two observations and the one with 0.75 *R*-squared value are identified on Figures 3 to 5, which provide plots of $\ln(t_{6.5})$ versus levels of salt, SPP and NaL, respectively, with linear regression lines, by temperature. For each of these points, it can be seen that, relative to other points with the same x-axis value, the value of $\ln(t_{6.5})$ is "separated" from the other values of $\ln(t_{6.5})$ in at least one of the figures. For example, in Fig. 3, for salt, two points are identified with a dark squares representing observations at 65°C, but corresponding predicted values are quite apart from the region where the other predicted values are for that temperature and within the region of the predicted value for 60°C. As a result of this analysis, these three points, rep-

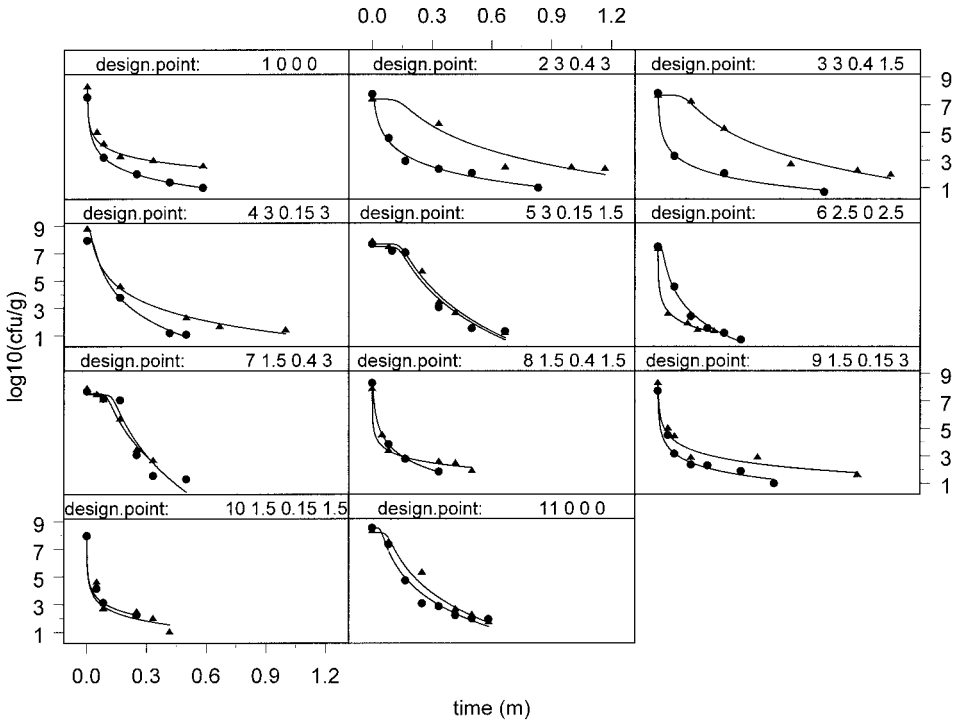

FIG. 2. Observed and fitted survival curves for the different combinations of values of salt, SPP, and NaL, for temperature = 71.1°C. The first number in the heading for the graphs is a design number designator; the following three values represent the salt, SPP, and NaL, values, respectively. For each design point there were two experiments; data points labeled with the same symbol are from the same experiment.

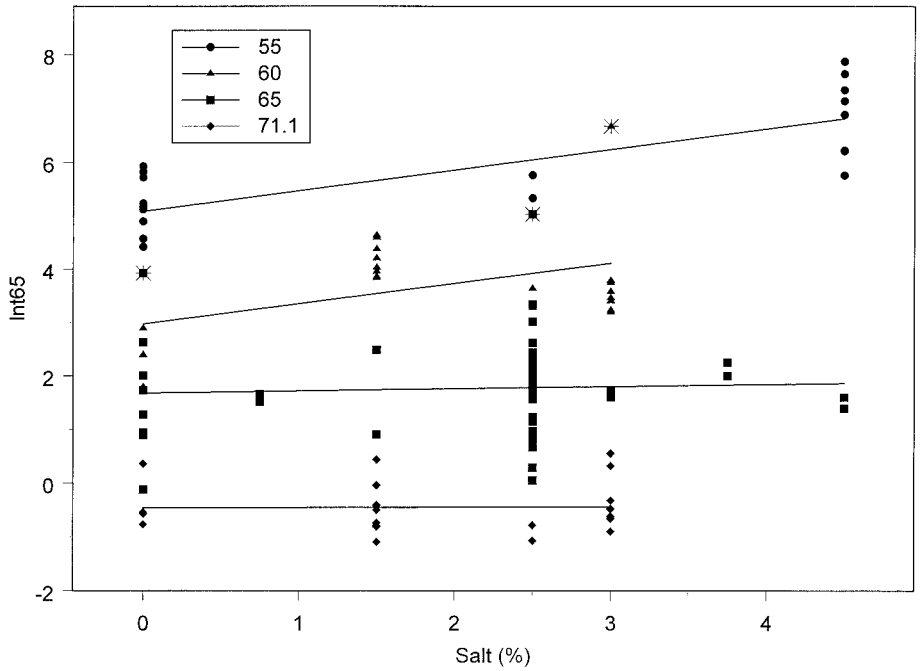

FIG. 3. Scatter plot of estimated natural log of times (m) needed to obtain a 6.5 lethality, $\ln(t_{6.5})$, from individual nonlinear regression (equation 1) versus levels of salt (%), with linear regression lines by temperature. Points excluded from analyses are indicated with asterisks superimposed on symbols.

resenting 3 survival curves and the data associated with them, were deleted, leaving data from 107 survival curves in the analysis.

An examination of the influence statistics of the stepwise regressions revealed one data point that was highly influential for predicting values of the parameter *a*, which can be seen on Fig. 5, at 55°C and an NaL concentration of 4.5% at about a value of $\ln(t_{6.5})$ of 8. The high degree of influence for this data

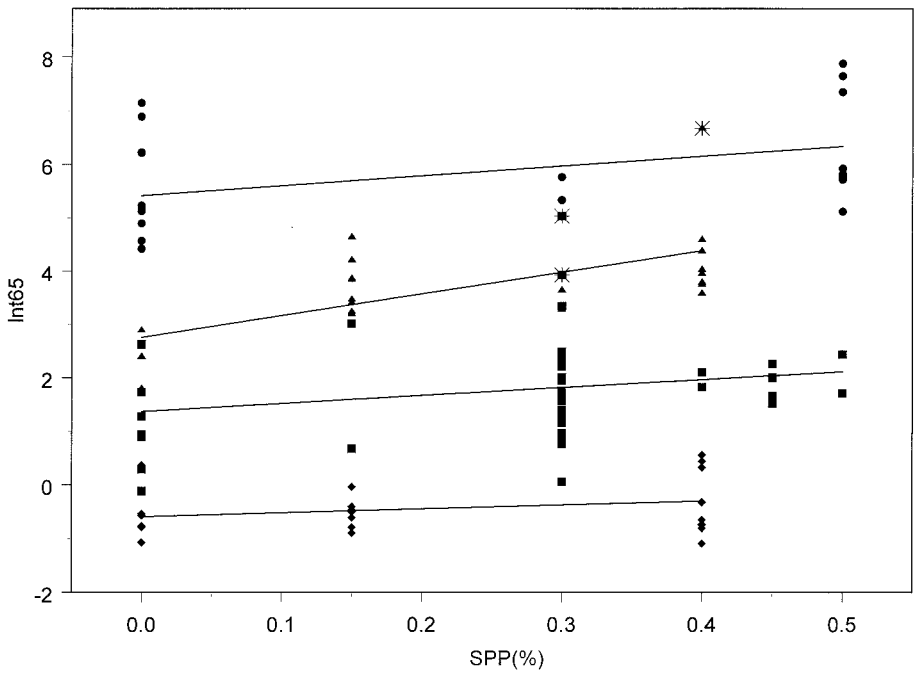

FIG. 4. Scatter plot of estimated natural log of times (m) needed to obtain a 6.5 lethality, $\ln(t_{6.5})$, from individual nonlinear regression (equation 1) versus levels of SPP, with linear regression lines by temperature. Points excluded from analyses are indicated with asterisks superimposed on symbols.

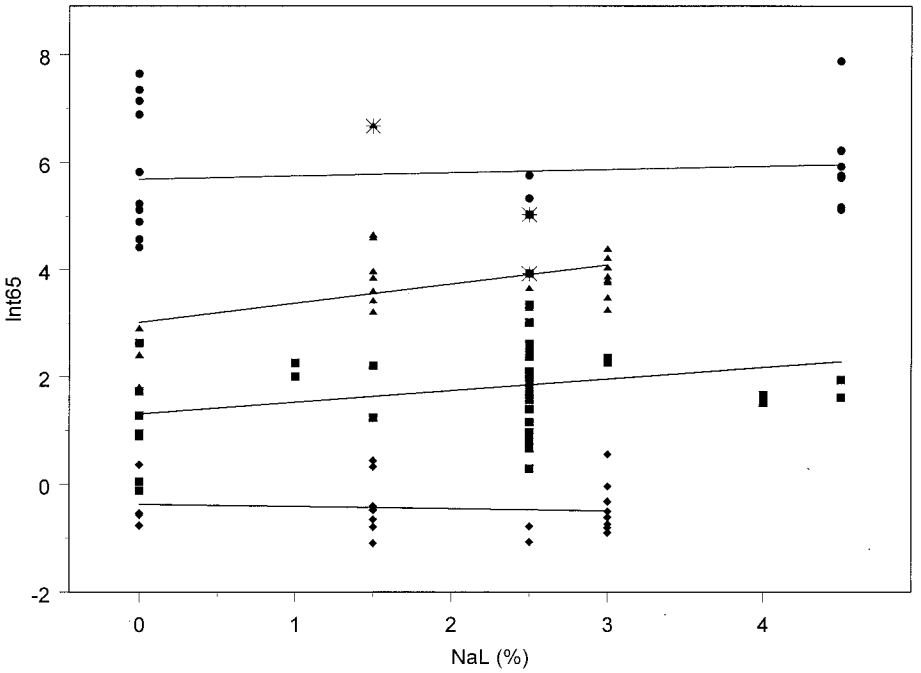

FIG. 5. Scatter plot of estimated natural log of times (minutes) needed to obtain a 6.5 lethality, ln($t_{6.5}$), from individual nonlinear regression (equation 1) versus levels of NaL, with linear regression lines by temperature. Points excluded from analyses are indicated with asterisks superimposed on symbols.

point is "caused" by the large difference between its value of ln($t_{6.5}$) and the value for its replicate data point, and by the location of the data point at the most extreme boundary of the region or range of values of the independent variables. Because of the high relative degree of influence, there is a strong argument to delete this data point, particularly so if its inclusion would actually change any general conclusion. However, this did not happen; the effect of including this point was to increase by slight amounts the RMSE and the standard error of prediction for the omnibus model. The standard deviation of the replicate values of ln($t_{6.5}$), while among the largest of the replicate standard deviations, was not the largest, so consequently, it was decided to leave the point in the analysis.

Using the results from the 107 survival curves in the stepwise regression, for ln($t_{6.5}$), the first variable selected was ln(temperature), followed by salt, the interaction of salt and ln(temperature), and the square of SPP. For the parameter *a*, the first variable to enter was ln(temperature), followed by the square of salt, the square of temperature, and the interaction of SPP and NaL, represented as the product of SPP and NaL. For the variable *b*, the only variable was the square of salt.

The fact that a function of salt entered the stepwise regression for *b* and a function of temperature did not needs further investigation. Figure 6 is scatter plot of the estimated values of *b* versus salt levels, with quadratic regression lines for each temperature. All but one value of b are greater than 1, the exception for 65°C. It is not clear that the values of *b* are not dependent on temperature; on the average, the highest values of *b* are for 55°C, with an average of 5.6; followed by 71.1°C, with an average of 5.0; then by 60°C, with an average of 4.9; and then 65°C, with an average of 3.8. The analysis of variance indicated a temperature effect, and when ln(*b*) is the dependent variable, ln(temperature) entered the stepwise regression first, followed by the square of temperature, interaction of salt and temperature, temperature, salt, and last the square of salt, which had a significance levels of 0.08. Consequently, for the omnibus model, it is not to be assumed that *b*, the coefficient of ln(time) in equation 1, is not dependent upon temperature. Furthermore, the figure shows an inconsistent dependency of the value of *b* on the salt level, where, for the three highest temperatures, the values of *b* are on the average increasing with salt level, with a convex shaped quadratic curve; however, for 55°C, the relationship is reversed (the quadratic curve is concave, where the maximum value is between 2 and 2.5% salt). However, this type of interaction: a concave relationship for one temperature and convex for the others, was not expected, and would, if representing a true relationship, imply a more complex model than anticipated. Rather, it was assumed that this pattern was a result of experimental variation.

Table 2 presents the means and standard deviations of the estimated values of ln($t_{6.5}$) from the derived 107 regressions using equation 1 for the 45 design combinations of this study. Included in this table are the mean of the estimated ln($t_{6.5}$), the standard deviation of these among the replicate experiments for each design combination, and the pooled standard error of these estimates due to regression, obtained by taking the square root of the sum of the weighted variances for the individual estimates ln($t_{6.5}$), with weight equal to the degrees of freedom of the regression. The correlations between these standard deviations and errors with the means of the ln($t_{6.5}$) were not significantly different from zero; thus, pooling variances over the row entries of the table provides a rough summary of the goodness-of-the fit of equation 1 for individual survival curves. The last row of the table thus includes the

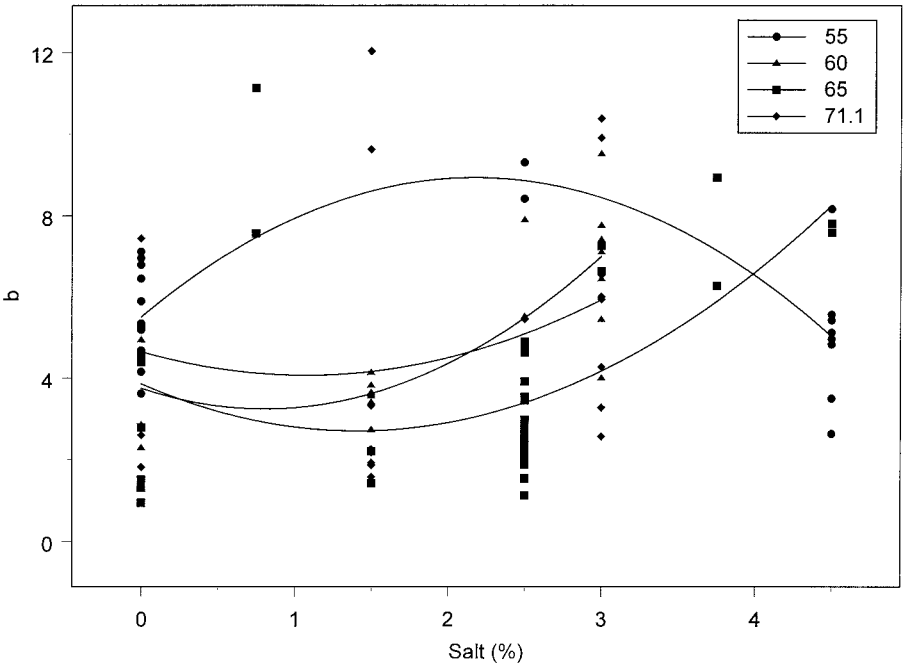

FIG. 6. Scatter plot of estimated values of parameter *b* in equation 1 from individual nonlinear regressions versus levels of salt, with quadratic regression lines by temperature.

pooled standard deviations, weighted by the number observations minus 1. The entry 0.641 in the last row of Table 2 in the column headed by between experiment standard deviation of $\ln(t_{6.5})$ can be used to compute, roughly, a probability range of estimated times from single experiments, by adding and subtracting an appropriate quantile of the standard normal distribution times 0.641 to the estimated $\ln(t_{6.5})$ and taking the exponential of the resultants. Thus, a 99% percent probability range would be almost factor of 30; for example, the range associated with an estimated time of 10 min would be 1.9 to 52 min. A good portion of this range is due to the error of the regression: the range for an estimated 10 min due to regression would 3.6 to 28 m, obtained using 0.401, the entry of the last row in the last column.

The between-experiment variance can be thought of as a sum of variance components: between replicate, within design combination ($n = 55$), and between repeated-design combinations. The between-repeated-design combination variance components is based on five design combinations for which replicates were repeated (from Table 2, top to bottom): three, two, three, five, and two times. The within-design combination variance component depends on 52 replicates, since three results were deleted. The analysis of variance on $\ln(t_{6.5})$ indicated a negative between repeated-design combination variance component; however, the highest five replicates accounted for 63% of the sum of the variances suggesting that the underlying distribution of results is not normal ($P = 0.10$, based on 10,000 simulations). A similar analysis was performed for $t_{3.0}$, the estimated time needed to achieve a 3.0 lethality. Here, the intra-repeated-design combination correlation was 86% indicating, relatively, a very high variance between repeated-design combinations. Consequently for the models, it is assumed that there is a nonzero between repeated-design variance component.

The above results and a close examination of Table 2 reveals that the NaL does not have consistent relationship with $t_{6.5}$ (note particularly the results for 55°C, rows 6 and 8). This can be seen from Table 3, where, assuming that the other three variables are constant, the number of times that the geometric mean of $t_{6.5}$, for a larger value of the 4th independent variable, is greater than that for a smaller value of the same independent variable is given for each temperature. For each temperature other than 65°C, there are 4 such comparisons for each variable; and for 65°C, there were 10 such comparisons. The same statistic is given for $t_{3.0}$, the estimated time needed to achieve a 3.0 lethality. As is evident from this table, with the exception of 65°C, for both $t_{3.0}$ and $t_{6.5}$ the percentage of times that the above increasing relationship holds for NaL is near 50%, whereas for salt and to a lesser extent SPP, the percentages are larger, with the notable exception for $t_{6.5}$ for salt at 60 and 65°C. The results indicate that while a relationship seems to

TABLE 3. Number of times there is an increasing relationship of the estimated times needed to achieve $t_{3.0}$ and $t_{6.5}$ for a given variable, holding three of the other variables constant

Temp (°C)	No. of comparisons	No. of times increase observed					
		NaCl		SPP		NaL	
		$t_{3.0}$	$t_{6.5}$	$t_{3.0}$	$t_{6.5}$	$t_{3.0}$	$t_{6.5}$
55	4	4	4	4	4	2	2
60	4	4	0	4	4	2	2
65	10	9	2	7	5	7	7
71.1	4	4	3	3	3	3	2

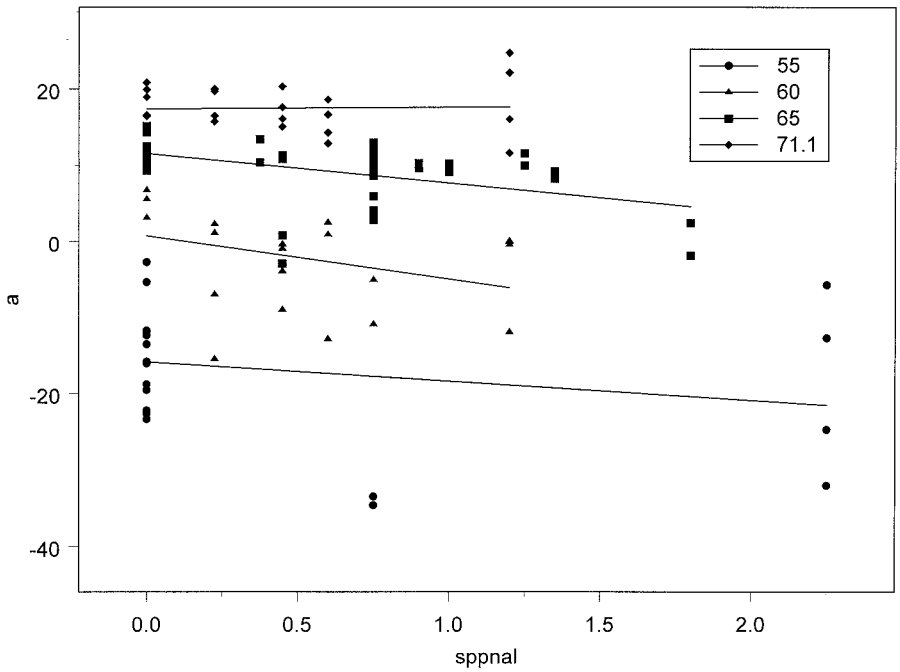

FIG. 7. Scatter plot of estimated values of parameter a in equation 1 from individual nonlinear regressions versus levels the product of SPP and NaL, with linear regression lines by temperature.

exist for lethalities with salt for low lethalities, and SPP, there does not appear to be as strong or as clear relationship of lethalities and NaL.

However, in the stepwise regressions, the interaction term of SPP and NaL was selected. The examination of the influence statistics that are part of the output did not reveal any particular observation that would be having an inordinate amount of influence on this interaction term. Furthermore, from a scatter plot (Fig. 7) of the estimated values of *a* versus the product of SPP and NaL, with linear regression lines by temperature, it is seen that three of the four linear regression lines—for temperatures 55 to 65°C—are decreasing and nearly mutually parallel, while the fourth line at 71.1°C is nearly horizontal. For the variable *b* there is a similar pattern. Thus, it is seen why the interaction term enters the regression, possibly reflecting some sort of synergistic effect of the two compounds on the lethality.

Secondary models. For the mixed linear effects model for fitting the logit transformation, $f(x) = \ln(10^{-x} - 1)$, where x is the $\log_{10}$ of the relative reduction, a nested error structure: temperature, combination within temperature, and replicate within experimental combination, was considered. Observations at time zero were deleted. Observations from the 107 experiments, excluding the 3 experiments identified above, were included in this analysis. Models were considered by adding or deleting parameters and were evaluated by considering the −2 ln(likelihood ratio), as described under "Statistical methods" section. The error structure assumed was nested, with design combination ($n = 55$), replicate (2) within design combination, considered as random factors. All variances and correlations were significantly different from zero, and when any of these parameters were eliminated from the model the likelihood ratio statistic was significant, at better than the 0.05 level. Three models were found to provide basically the same likelihood: (i) the model given in Table 4 that presents the estimated fixed parameter values of the model; (ii) the same model except the interaction of salt and ln(temperature) is used instead of salt [for this model, the value of −2 ln(likelihood function) is only slightly larger, by 0.05, than that of the

TABLE 4. Estimates of parameter values used for predicting lethality

Effect	Estimate	SE	df	*t*	Pr > |*t*|
Intercept	−4,300.19	998.72	56	−4.31	<0.0001
ln(temp)	1,963.14	482.80	55.7	4.07	0.0002
ln(temp) · ln(temp)	−222.83	58.3306	55.4	−3.82	0.0003
Salt	−0.6028	0.2489	44.5	−2.42	0.0196
SPP · NaL	−1.7392	0.6333	45	−2.75	0.0086
ln(tt)	1,224.29	470.67	52.2	2.60	0.0121
ln(temp) · ln(tt)	−587.44	227.93	52.3	−2.58	0.0128
ln(temp) · ln(temp) · ln(tt)	70.6700	27.5867	52.4	2.56	0.0133

[a] Based on equation 1. From linear mixed effect model (PC-SAS) based on the maximum-likelihood method. The variable ln(tt) represents ln(time), where time is in minutes, and ln(temp) is ln(temp), where temperature is in degrees Celsius.

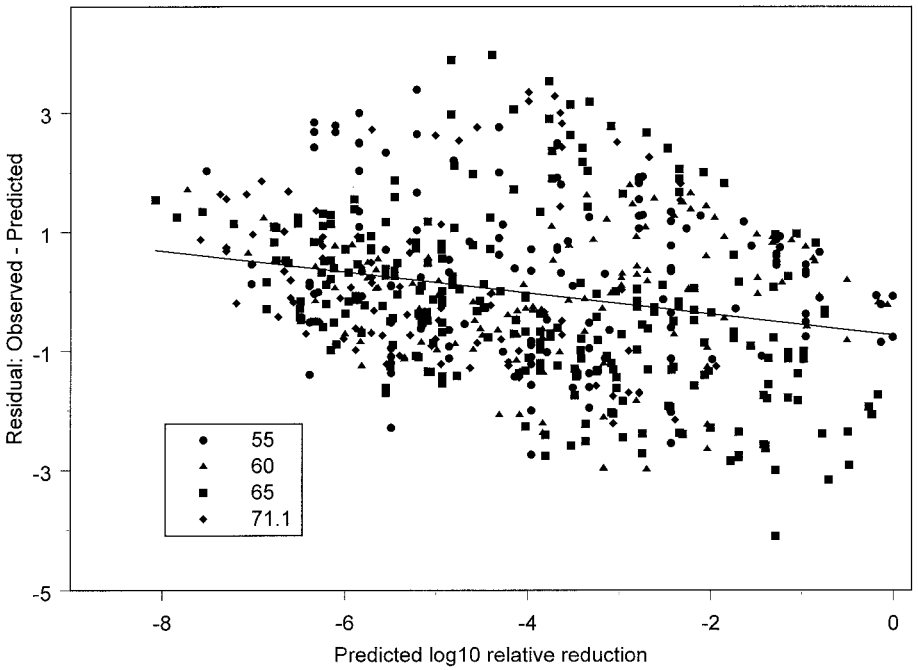

FIG. 8. Scatter plot of residuals versus predicted $\log_{10}$ relative reductions obtained from omnibus model (Table 4) with linear regression line.

model of Table 4, and the average standard error of prediction increases only slightly, from 0.4232 to 0.4236]; and (iii) instead of salt and the interaction of SPP and NaL, the terms SPP and NaL are used [for this model, the value of -2 ln(likelihood function) is slightly less, by about 1, than that of the model of Table 4, and the average standard error of prediction decreases slightly, from 0.4232 to 0.4223]. The model of Table 4 is chosen for further analysis insofar as it does include the salt variable, whereas the third model does not, and choosing this model over the second model was just a matter of choice, based on the stepwise regression for the variable a where salt rather than interaction of salt with temperature was selected. Furthermore, adding other terms, such as salt, SPP, or the square of SPP did not decrease the likelihood function significantly. Only adding a term involving the square of salt and the interaction of the square of salt and temperature for b did the model goodness-of-fit criterion improve significantly ($P = 0.03$), even though the average of the standard errors of predictions increased to 0.4635. However, as discussed above in connection with Fig. 6, the interaction term was not considered; and when deleting that term, the significance of the salt squared term vanished. Models including the parameter c of equation 2, suggesting that there would be a asymptotic nonzero D value, were also considered. These models also met our 0.05 significance level criterion (e.g., 0.01) when compared to the model of Table 4, but provided, on the average, higher standard errors of predictions, and, more important, negative estimates of c for some conditions, contrary to the restriction that $c > 0$. Consequently the model of Table 4 is chosen for further analysis, though it being selected does not imply that terms not in the model do not have an effect on the lethality—in particular, that there is not an interaction between salt and temperature or there is not, asymptotically, a nonzero D value.

For the model of Table 4, the standard errors of the predicted $\log_{10}$ of the relative reduction range up to 1 $\log_{10}$, the higher values for the higher and lower temperatures of 55 and 71.1°C. Figure 8 is the plot of the residuals of the predicted $\log_{10}$ of the relative reduction versus the predicted values. As is seen, there is a trend in the residuals; this type of trend existed for all the models discussed above, and is a result of the incompleteness of the design, the transformation, and the existence of the variance components. When examining the residuals of the predicted logit, treating the random effect parameters as fixed, the correlation is 0.08($P = 0.04$). For the model of Table 4, the estimated mean values of b, with standard errors, are: at 55°C, 5.08 (0.43); at 60°C, 3.79 (0.28); at 65°C, 3.53 (0.26); and at 71.1°C, 4.33 (0.45). From the mixed effects model, the between-experiment standard deviation of b is estimated to be approximately 1.41, so that, for example, at 65°C, the 95% probability interval is estimated to be $3.53 \pm 2.82 = (0.71, 6.35)$, ignoring the uncertainty of the estimated values. Thus, while it is expected that survival curves will have shoulders, the model predicts that some experiments will not have shoulders, a consequence of experimental variation.

Figure 9 presents the fitted survival curves for the 45 distinct design combinations studied, together with the observed $\log_{10}$ relative reductions. The x axis represents a measure of time that is normalized by dividing the actual time by the estimated time to achieve a 6.5 lethality derived from the omnibus model. The graphs also include curves representing estimated 90% upper and lower probability bounds for the obtain lethalities depicting the expected probability range of survival curves for single experiments, derived using the estimated variance components of the mixed effect model. With a few exceptions, the curves derived from the omnibus model provide a reasonable fit or coverage of the observed data points.

As mentioned in the previous sections, of particular impor-

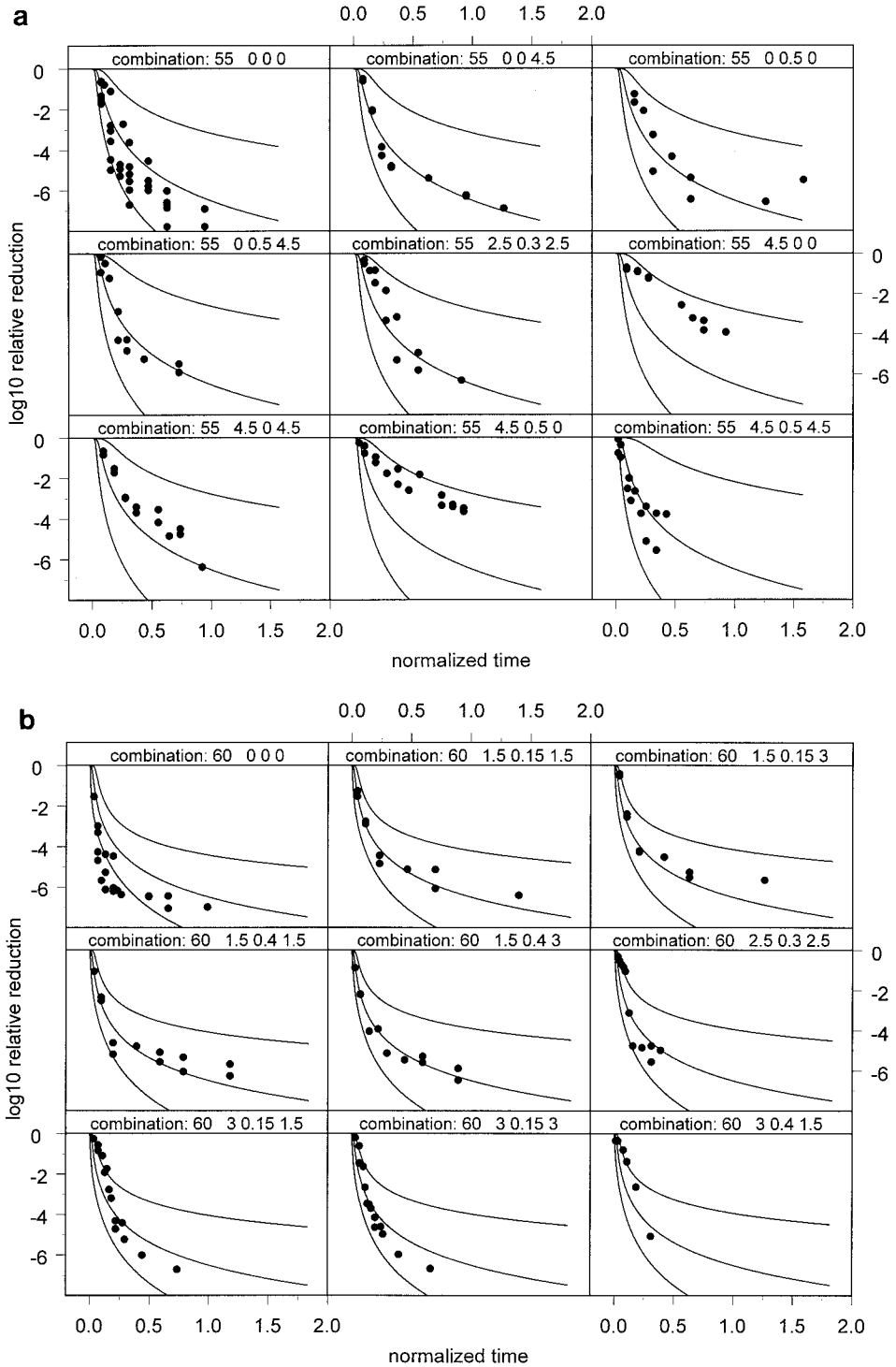

FIG. 9. Observed and fitted survival curves derived from the omnibus model (Table 4) for the 45 combinations of values of temperature (in degrees Celsius) and salt, SPP, and NaL concentrations (percentages) studied (Table 2). The middle line represents the predicted survival curve, and the two outer lines represent 90% upper and lower probability bounds, depicting the expected probability range of survival curves for single experiments. The time axis has been normalized by dividing the actual time by the predicted time to obtain a 6.5 lethality derived from the omnibus model. Each panel (a to e) shows graphs for nine combinations.

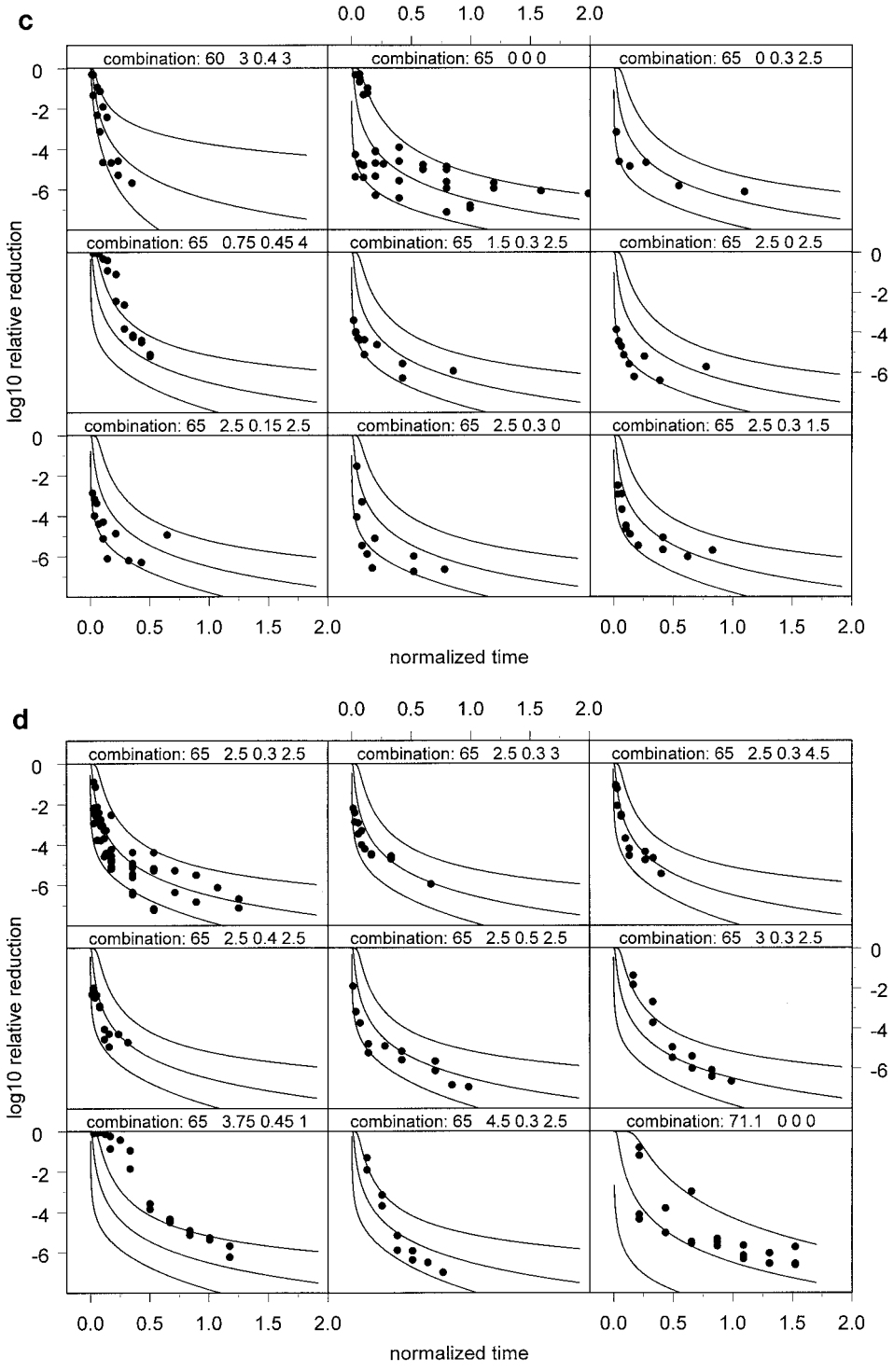

FIG. 9—*Continued.*

tance is the prediction of the times, $t_{6.5}$, needed for a 6.5 $\log_{10}$ relative reduction, using equation 4. The estimated times for obtaining a 6.5 lethality derived from the omnibus model compare reasonably well (with a few exceptions) with those obtained directly from the individual nonlinear regressions of equation 1 given in Table 2. Figure 10 is a plot of the difference between the mean estimated natural logarithm of the times, reported in Table 2, and the corresponding estimate derived from the omnibus regression, versus the average of the two estimates. However, the standard errors of predictions from the omnibus model are large.

Thus, instead of using the omnibus model for estimating the

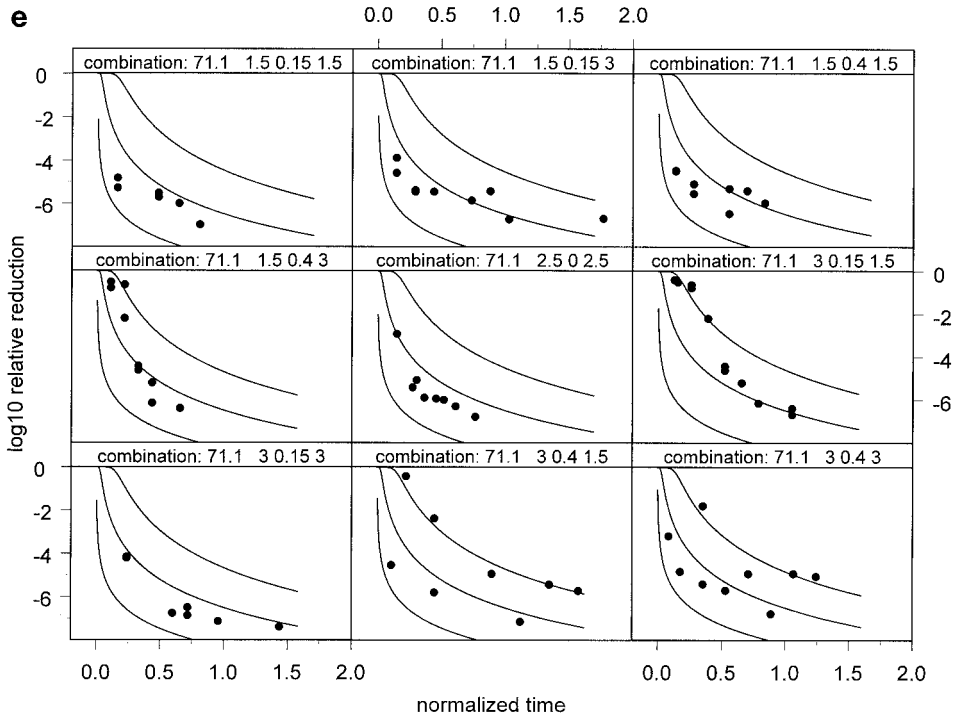

FIG. 9—*Continued.*

times needed to obtain a 6.5 lethality, a regression with dependent variable $\ln(t_{6.5})$ obtained from the 107 individual regressions of equation 1 can be used directly. With few exceptions, the replicate standard deviations are small compared to the magnitude of the residuals; thus, a simple linear regression was used with the maximum likelihood estimation method, where the dependent variable was the mean of the estimated $\ln(t_{6.5})$ for each design combination (55 in total). The selected model is given in Table 5. Adding variables did not significantly improve the model, when using the chi-square approximation to

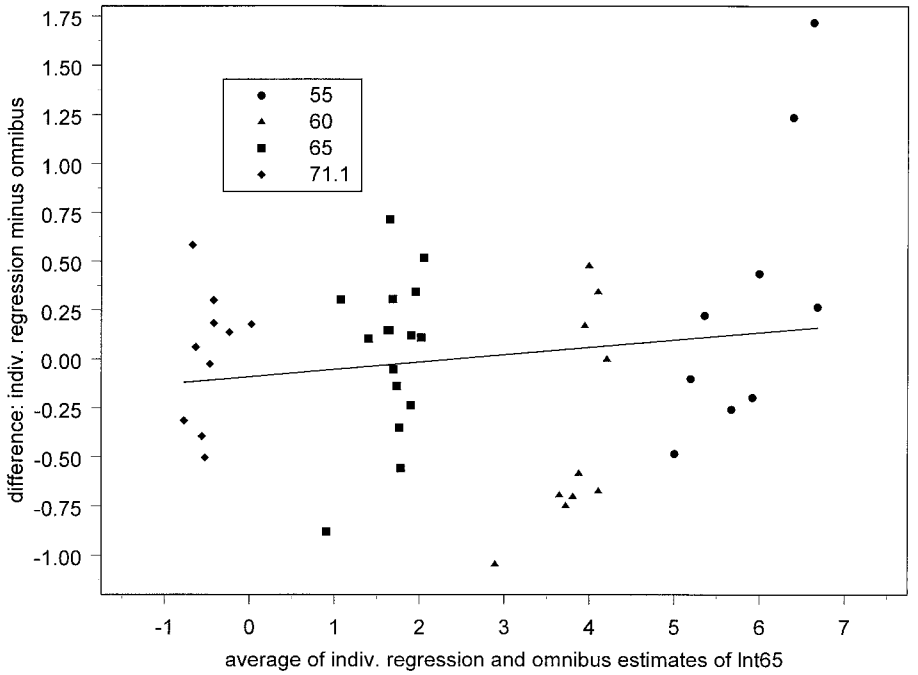

FIG. 10. Scatter plot of difference between mean of estimated natural logarithm of time needed to obtain a 6.5 lethality, $\ln(t_{6.5})$, obtained from individual regressions (Table 2) and omnibus model (Table 4) versus average of the two estimates.

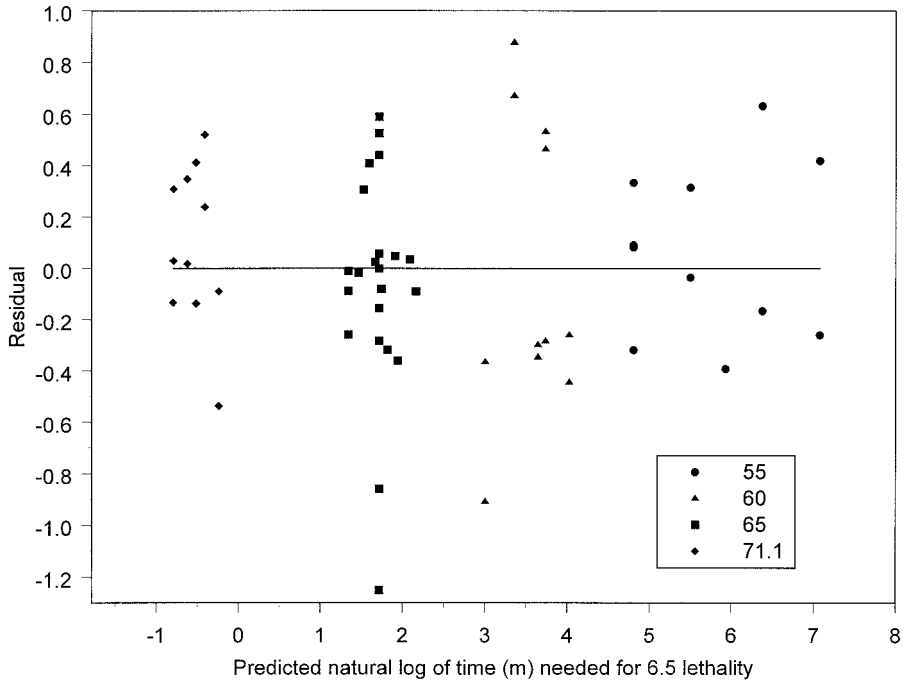

FIG. 11. Scatter plot of residuals versus predicted natural logarithm of the time needed to obtain a 6.5 lethality, $\ln(t_{6.5})$, using the model in Table 5, where the dependent variable is the $\ln(t_{6.5})$ derived from individual regressions of 107 observed survival curve.

L = −2 ln(likelihood ratio) statistic as a criterion. Using SPP instead of the square of SPP increased L by 0.5; adding the square of ln(temperature) decreased L by 1.4; adding a linear term for SPP decreased L by 0.1; adding NaL and the interaction of SPP and NaL decreased L by 1.7; using the interaction of SPP and ln(temperature) instead of the interaction of salt and ln(temperature) increased L by 14.7. The linear regression line of the residuals versus the predicted values of $\ln(t_{6.5})$ is virtually flat (Fig. 11). The standard errors of the predicted $\ln(t_{6.5})$ for the data range from about 0.065 to 0.20, however, for product at 71.1°C, a salt concentration of 4.5%, and an SPP concentration of 0% the standard error is 0.25. The CV of the estimated times can be approximated as 100% times the standard error of the estimated ln(times). An estimated CV of 20% implies that a 99% confidence interval of the estimate of the expected times needed to obtain a 6.5 lethality ranges by a factor of about 3 (based on 50 df); thus, for example, an estimated time of 10 min would have 99% confidence interval of 5.8 to 17.1 m; an estimated CV of 30% implies that a 99% confidence interval ranges by a factor of about 5; thus, an estimated time of 10 min would have 99% confidence interval of 4.5 to 22 min. Table 6 gives the estimated times to obtain a 6.5 lethality obtained from the individual nonlinear regressions using equation 1 (Table 2); the linear mixed effects regression using these estimated times (actually the natural log of them); and the omnibus model, given in Table 4. Included are the estimated CV's obtained from the latter two regressions, estimated by multiplying the standard error of the estimated ln(time).

TABLE 5. Estimates of parameter values using linear model for predicting the natural logarithm of the (minimal) times needed to obtain a 6.5-$\log_{10}$ relative reduction of *Salmonella*[a]

Effect	Estimate	SE	df	*t*	Pr > |*t*|
Intercept	87.9325	4.2538	50	20.67	<0.0001
ln(temp)	−20.7426	1.0325	50	−20.09	<0.0001
Salt	7.5475	1.7342	50	4.35	<0.0001
ln(temp) · salt	−1.7962	0.4232	50	−4.24	0.0001
SPP · SPP	2.7767	0.7212	50	3.85	0.0003

[a] The dependent variable was the natural log of the estimated time obtained from individual nonlinear regression of equation 1. See footnote *a* to Table 4 for abbreviations.

The predicted effect of SPP on the predicted times based on model using the individual estimates, $\ln(t_{6.5})$, is clear: the higher the level of SPP, the more time it takes to achieve a 6.5 $\log_{10}$ relative reduction. The interaction of temperature and salt is statistically significant ($P = 0.0001$). Example predictions: for a temperature = 60°C, salt = 0%, SPP = 0%, the predicted time is 20.2 m, with a CV of 10.8%; for 60°C, 4.5% salt, and 0% SPP, the predicted time is 48.2 min with CV of 15.2%; for 71.1°C (160°F), 0% salt, and 0% SPP, the predicted time is 0.60 m, with CV of 18.3%; for 71.1°C, 4.5% salt, and 0% SPP, the predicted time is 0.36 m, with CV of 24.7%. Increasing salt from 0 to 4.5% at 60°C increases the time needed to obtain a 6.5 $\log_{10}$ relative reduction by a 2.4 factor (CV = 18.6%), significant at <0.0001; at 63.5% the factor is about 1.5, significant at the 0.06 level, but at 71.1°C the difference in the times is not significant ($P = 0.18$).

However, for lower lethalities, the salt effect seems to be more pronounced. A similar analysis as above was performed for the estimated time to obtain a 3 lethality, $t_{3.0}$. The "best" model included ln(temperature), the square of ln(temperature), salt (coefficient of 0.369, $P = 0.002$), and SPP (coefficient of 1.90; $P = 0.05$). Interaction terms or terms involving NaL

TABLE 6. Estimated times from individual regressions (Table 2), regression using these times (Table 5), and omnibus mixed effects regression (Table 4)

Temp (°C)	NaCl (%)	SPP (%)	NaL (%)	Time/min to obtain 6.5 lethality from:			CV (%) of estimated time from regression of individual estimates	Approx.[a] CV (%) of estimated time from omnibus regression
				Individual nonlinear regressions	Regression from estimated times	Omnibus regression		
55.0	0.00	0.00	0.0	116.93	122.75	189.96	15.56	61.08
55.0	0.00	0.00	4.5	171.49	122.75	189.96	15.56	61.08
55.0	0.00	0.50	0.0	237.09	245.75	189.96	19.28	61.08
55.0	0.00	0.50	4.5	336.95	245.75	410.53	19.28	71.73
55.0	2.50	0.30	2.5	255.14	377.67	330.44	10.90	65.02
55.0	4.50	0.00	0.0	1,113.4	591.87	324.07	19.96	66.48
55.0	4.50	0.00	4.5	501.33	591.87	324.07	19.96	66.48
55.0	4.50	0.50	0.0	1,803.4	1,184.9	324.07	20.00	66.48
55.0	4.50	0.50	4.5	913.41	1,184.9	700.37	20.00	74.66
60.0	0.00	0.00	0.0	10.68	20.19	30.40	10.79	39.67
60.0	1.50	0.15	1.5	69.01	28.72	42.81	7.83	39.87
60.0	1.50	0.15	3.0	56.22	28.72	47.48	7.83	40.26
60.0	1.50	0.40	1.5	71.72	42.08	50.87	8.68	40.72
60.0	1.50	0.40	3.0	66.98	42.08	67.04	8.68	44.01
60.0	2.50	0.30	2.5	31.67	42.03	63.93	6.76	42.35
60.0	3.00	0.15	1.5	27.14	38.39	54.38	9.70	42.30
60.0	3.00	0.15	3.0	28.49	38.39	60.31	9.70	42.47
60.0	3.00	0.40	1.5	36.02	56.23	64.61	8.59	42.78
60.0	3.00	0.40	3.0	43.35	56.23	85.15	8.59	45.45
65.0	0.00	0.00	0.0	3.41	3.84	2.52	11.97	25.31
65.0	0.00	0.30	2.5	7.43	4.93	3.64	12.12	26.83
65.0	0.75	0.45	4.0	4.88	6.99	6.93	13.55	36.41
65.0	1.50	0.30	2.5	5.45	5.31	4.70	6.48	24.30
65.0	2.50	0.00	2.5	4.27	4.34	3.85	9.32	25.64
65.0	2.50	0.15	2.5	6.29	4.62	4.63	8.23	24.46
65.0	2.50	0.30	0.0	1.60	5.58	3.85	6.48	25.64
65.0	2.50	0.30	1.5	5.57	5.58	4.81	6.48	24.44
65.0	2.50	0.30	2.5	5.27	5.58	5.57	6.48	25.16
65.0	2.50	0.30	3.0	10.07	5.58	6.00	6.48	25.94
65.0	2.50	0.30	4.5	5.91	5.58	7.49	6.48	29.74
65.0	2.50	0.40	2.5	7.11	6.77	6.30	8.09	26.61
65.0	2.50	0.50	2.5	7.95	8.70	7.13	13.06	28.73
65.0	3.00	0.30	2.5	5.28	5.72	6.07	7.94	26.31
65.0	3.75	0.45	1.0	8.40	8.11	5.95	12.38	28.55
65.0	4.50	0.30	2.5	4.48	6.16	7.84	14.51	32.01
71.1	0.00	0.00	0.0	0.69	0.60	0.38	18.32	22.08
71.1	1.50	0.15	1.5	0.55	0.54	0.52	10.55	19.13
71.1	1.50	0.15	3.0	0.76	0.54	0.56	10.55	18.92

[a] CVs are approximated by 100% times the standard error of estimated ln(time).

did not improve the fit of the model with the data; however, as mentioned above, this does not mean that such terms are not important. The point of mentioning this analysis is to support the results from Table 3 that salt has an effect for low lethalities, whereas the model of Table 5 for $t_{6.5}$ suggests that, at higher temperature, the salt effect seems to be less pronounced for larger lethalities, or, depending upon temperature, possibly reversed, though, as seen above for the result at the end of the last paragraph for 71.1°C, the estimated effect of decreasing the heat resistance was not statistically significant.

DISCUSSION

D values are used often in reporting kinetic results of inactivation studies, but many researchers (3, 6, 7) have reported nonlinear curves for *Salmonella* spp. Murphy et al. (13) fit nonlinear curves that have initial lag times, but reported only *D* values. Even in papers that report *D* values, it is often stated that the linear portion of the survival curve was used, implying that the actual survival data indicated some type of nonlinear shape. Thus, comparisons of predicted times needed to obtain specified lethalities using nonlinear models with predicted times based on reported *D* values may not be appropriate; it might be that the shoulders and the tails of the survival curves are affected by the concentrations of the factor being studied, for example, as seems to be the case regarding the effect of fat concentrations on the heat resistance of *Salmonella* in meat and poultry (7, 9). In this work, the survival curves were nonlinear and displayed tailing, and thus it was not possible to report *D* values and use them for direct comparisons with other published results. Hence, the comparisons that are presented below need to be understood as only rough approximations.

There have been many studies showing that high concentrations of salt increase heat resistance of *Salmonella* (5). For example, results reported by Mañus et al. (11), seem to agree, in part, with our finding that increasing levels of salt increases the heat resistance of *Salmonella* at lower temperatures. In the Mañus et al. (11) paper, the effects of salt concentrations on the heat resistance of *Salmonella enterica* serovar Typhimurium was studied in broth at 58°C. Results

given in that paper (in terms of *D* values) indicated that increasing levels from 0 to 4.5% would double the *D* value, implying that the time needed to obtain a fixed lethality would double. For the model given in Table 5, to obtain a 6.5-$\log_{10}$ reduction, at 58°C when increasing the salt from 0 to 4.5% and assuming that the concentration of SPP is 0%, the estimated time needed increased 3.1-fold, with CV of 19.8%, so that a lower 95% confidence limit is 2.3-fold, which is slightly higher than the estimated factor of 2 reported in the work of Mañus et al. (11).

Blackburn et al. (3) also reported that higher levels of salt increased heat resistance, but noted that beyond 3.5% the heat resistance stayed about the same. The highest value for our study was 4.5% so that the linear effect for a given temperature that is used in the model developed within this paper is not inconsistent with the findings in Blackburn et al. (3). A predicted *D* value for *Salmonella* serovar Enteritidis of 0.9 min at 60°C for beef with a salt level of 0.23% (wt/wt, aqueous phase) was reported (3) (Table 6). Using this *D* value, to obtain a 6.5-$\log_{10}$ relative reduction of *Salmonella*, the estimated time needed would be 5.85 min. However, in that same paper, a graph is presented that shows that the estimated times needed to obtain a 5-$\log_{10}$ relative reduction from the nonlinear model that was used in that paper is about twice as large as those estimated using the predicted *D* values. We should point out that the model used in the Blackburn paper had the property that the probability of viable cells did not approach 0 as *t* approaches infinity. It seems reasonable to assume that the Blackburn, et al. (3) model reflected the behavior of their observed survival curves for relatively large times, thereby possibly being relatively "flat" with extensive tailing. Thus, it is quite possible that, for a 6.5 $\log_{10}$ relative reduction, the ratio of the of the predicted times obtain from the nonlinear curves versus the estimated times obtained from the *D* values would be substantially larger than the estimated value of 2 for the ratio for obtaining a 5 lethality; the ratio for a 6.5 lethality could be more than 3. If the ratio were 3, then the estimated time needed to obtain a 6.5 $\log_{10}$ would be 17.6 min. From the model given in Table 5 (assuming SPP = 0%, salt = 0.23%, and temperature = 60°C), the estimated time is 21.1 m, with an error CV of 10.3%, and thus a lower 95% confidence bound of the needed time is about 17.8 min. Thus, the different estimates may not be statistically different.

Conclusion. The results of data analysis indicated that salt and sodium pyrophosphate (SPP) significantly affect the heat resistance of *Salmonella* spp. Increasing the level of SPP increases the heat resistance. Increasing the salt levels increases the heat resistance for lower temperatures (<63.5°C), but for higher temperatures and large lethalities, salt levels did not significantly affect the heat resistance. NaL did not seem to affect the heat resistance of *Salmonella* as much as the effects induced by the other variables studied.

The survival curves were convex. An omnibus model, assuming nonlinear survival curves, was developed for predicting the obtained lethality when cooking beef for a fixed amount of time at a fixed temperature between 55°C (131°F) and 71.1°C (160°F), where the beef matrices contain concentrations of salt between 0 and 4.5%; SPP between 0 and 0.5%; and NaL between 0 and 4.5%. The selected model included terms involving salt and the interaction of SPP and NaL. While the former term is not surprising, the latter term, by itself, without the presence of terms for SPP and NaL, presents difficulties in explaining the model: is there is a synergistic effect of the two compounds on lethality, at least for relatively small times, or is the statistical significance of this interaction term alone, without terms for SPP or NaL, just a fluke and that there really are SPP and NaL effects that were masked due to variability which could have only been detected in this study when the two compounds were both present. Further research is needed to clarify this.

For the omnibus model, however, the standard errors of prediction are large. Since there is special interest of the times needed to obtain a 6.5 lethality, a model was developed for predicting the times needed to obtained a lethality of 6.5 $\log_{10}$, using directly the estimated times to achieve a 6.5 lethality obtained from regressions of the individual survival curves. For the latter model, the CV of predicted times range from about 7 to 30%. The results indicate that at 71.1°C, the times needed to obtain a 6.5-$\log_{10}$ lethality could exceed 0.5 min.

The derived estimated times needed to obtain a 6.5-$\log_{10}$ lethality seem to be higher than predictions derived from reported *D* values in the published literature. A contributing reason for this could be due to the nonlinear survival curves. Predictions based solely on *D* values from the "linear" portion of the survival curves could be biased because of tailing of the survival curves for large times and because of the lag times—shoulders of the survival curves for small times before the linear kinetic inactivation begins. However, many researchers have reported nonlinear survival curves for *Salmonella*, and thus predicted times for obtaining specified lethalities need to be based on models of these types of curves and not on *D* values.

In addition, the standard errors or CVs of the estimated lethalities for a given time or predicted times needed to obtain a given lethality seemed rather large, in some cases, exceeding 20%, giving rise to rather large confidence intervals of estimated values. For example, based on the 50 df of the model of Table 5, an estimate of an expected 10 m, with a CV error of 20%, implies a 99% confidence interval covering 5.8 to 17.1 min. The confidence intervals do not include the variability that may arise from slight misspecifications of conditions, so that in actuality, to assure that processes would be meeting lethality objectives, larger upper bounds might be needed. While no standards of predictions have been established by professional organization, we suggest that, for omnibus models that need to satisfy multiple needs, the CV's of estimates of the expected values of times to achieve specific lethalities should not be much larger than 10%, so that confidence intervals would not be "too" wide. Consequently, for these types of studies more observations are needed, perhaps, more than two or three times as many as in this study. More research is needed to clarify the conditions that create nonlinear curves and to develop models for them.

APPENDIX

Shown in Table A1 are estimated parameters from individual regressions (equation 1).

TABLE A1. Estimated parameters from individual regressions

Temp (°C)	Salt (%)	SPP (%)	NaL (%)	Rep	No. of obs	RMSE	Time (min)[a]	CV of time (%)	*a*	*b*
55	0.0	0.00	0.0	1	5	0.593	133.23	49.27	−2.707	3.613
55	0.0	0.00	0.0	2	7	0.745	133.34	47.25	−5.347	4.152
55	4.5	0.50	4.5	1	8	0.314	2,633.7	44.77	−5.729	2.628
55	4.5	0.50	4.5	2	8	0.340	316.78	11.43	−32.09	8.172
55	4.5	0.50	0.0	1	10	0.071	2,091.4	7.80	−11.80	3.500
55	4.5	0.50	0.0	2	8	0.377	1,555.2	48.14	−22.68	5.123
55	4.5	0.00	4.5	1	8	0.211	498.05	12.79	−15.83	4.958
55	4.5	0.00	4.5	2	8	0.616	504.63	31.43	−18.79	5.424
55	4.5	0.00	0.0	1	7	0.358	980.69	34.39	−23.37	5.565
55	4.5	0.00	0.0	2	6	0.312	1,264.1	29.58	−19.54	4.832
55	2.5	0.30	2.5	1	7	0.247	316.51	7.37	−33.50	8.418
55	2.5	0.30	2.5	2	7	0.477	205.67	13.22	−34.62	9.310
55	0.0	0.50	4.5	1	6	0.599	374.03	38.10	−12.73	4.675
55	0.0	0.50	4.5	2	7	0.682	303.54	26.00	−24.76	6.951
55	0.0	0.50	0.0	1	5	1.166	166.77	60.34	−12.36	5.340
55	0.0	0.50	0.0	2	7	0.726	337.05	48.66	−11.74	4.588
55	0.0	0.00	4.5	1	7	0.673	167.25	35.20	−12.08	5.283
55	0.0	0.00	4.5	2	7	0.576	175.83	29.66	−11.82	5.181
55	0.0	0.00	0.0	1	6	0.376	96.10	17.81	−11.93	5.890
55	0.0	0.00	0.0	2	7	0.843	82.91	33.14	−13.52	6.449
55	0.0	0.00	0.0	1	7	0.628	96.53	24.38	−16.04	6.785
55	0.0	0.00	0.0	2	7	0.303	187.10	16.45	−22.23	7.110
60	0.0	0.00	0.0	1	6	0.547	10.92	62.12	9.523	2.277
60	0.0	0.00	0.0	2	6	0.492	18.00	45.16	6.759	2.840
60	3.0	0.40	3.0	1	8	0.304	44.21	17.95	−11.93	7.099
60	3.0	0.40	3.0	2	8	0.465	42.50	33.69	−0.010	3.994
60[b]	3.0	0.40	1.5	1	5	0.808	790.62	434.40	2.821	1.820
60	3.0	0.40	1.5	2	7	0.332	36.02	15.56	−12.84	7.757
60	3.0	0.15	3.0	1	8	0.344	25.35	14.09	−8.984	7.409
60	3.0	0.15	3.0	2	8	0.302	32.02	15.02	−3.892	5.440
60	3.0	0.15	1.5	1	8	0.369	24.44	13.36	−15.45	9.516
60	3.0	0.15	1.5	2	8	0.567	30.14	23.33	−6.951	6.435
60	2.5	0.30	2.5	1	7	0.429	26.41	14.46	−10.86	7.890
60	2.5	0.30	2.5	2	6	1.023	37.96	55.20	−5.057	5.506
60	1.5	0.40	3.0	1	6	0.445	79.71	39.24	0.062	3.404
60	1.5	0.40	3.0	2	8	0.322	56.29	22.33	−0.402	3.813
60	1.5	0.40	1.5	1	7	0.543	98.49	60.91	2.508	2.714
60	1.5	0.40	1.5	2	7	0.716	52.23	54.07	0.911	3.553
60	1.5	0.15	3.0	1	5	0.557	47.25	44.65	−0.957	4.130
60	1.5	0.15	3.0	2	7	0.588	66.90	45.46	−0.338	3.641
60	1.5	0.15	1.5	1	7	0.501	46.26	37.64	1.147	3.604
60	1.5	0.15	1.5	2	6	0.496	102.94	54.75	2.331	2.727
60	0.0	0.00	0.0	1	5	0.660	10.98	43.65	3.139	4.935
60	0.0	0.00	0.0	2	7	0.841	6.03	42.74	5.604	5.213
65	0.0	0.00	0.0	1	6	0.288	0.89	57.17	15.121	1.306
65	0.0	0.00	0.0	2	7	0.175	13.88	69.39	12.488	0.942
65	4.5	0.30	2.5	1	7	0.257	4.97	8.79	2.803	7.586
65	4.5	0.30	2.5	2	5	0.429	4.04	15.91	4.066	7.809
65	3.0	0.30	2.5	1	6	0.683	4.98	26.10	3.324	7.254
65	3.0	0.30	2.5	2	7	0.196	5.59	7.87	3.552	6.631
65	2.5	0.50	2.5	1	6	0.366	5.53	47.87	11.578	1.982
65	2.5	0.50	2.5	2	8	0.246	11.44	35.72	9.968	2.051
65	2.5	0.40	2.5	1	7	0.654	6.19	101.22	10.223	2.603
65	2.5	0.40	2.5	2	8	0.305	8.18	40.44	9.178	2.755
65	2.5	0.30	4.5	1	7	0.384	5.03	37.87	9.252	3.537
65	2.5	0.30	4.5	2	8	0.368	6.95	31.40	8.265	3.456
65	2.5	0.30	3.0	1	7	0.335	10.48	63.17	9.657	2.260
65.0	2.50	0.30	3.0	2	8	0.216	9.67	32.83	10.271	2.070
65.0[a]	2.50	0.30	2.5	1	5	0.253	152.26	220.16	10.831	0.823
65.0	2.50	0.30	2.5	2	7	0.414	4.77	35.79	10.315	2.976
65.0	2.50	0.30	2.5	1	7	0.273	6.99	15.65	5.967	4.628
65.0	2.50	0.30	2.5	2	8	0.247	10.75	46.53	10.522	1.872
65.0	2.50	0.30	2.5	1	7	0.374	2.13	21.47	11.464	4.639
65.0	2.50	0.30	2.5	2	6	0.420	2.62	28.63	10.309	4.832
65.0	2.50	0.30	2.5	1	7	0.588	2.39	32.60	10.704	4.898
65.0	2.50	0.30	2.5	2	8	0.440	7.41	51.02	9.318	2.821
65.0	2.50	0.30	2.5	1	7	0.259	3.16	17.23	9.583	4.682
65.0	2.50	0.30	2.5	2	7	0.299	28.23	66.20	8.615	1.902

Continued on facing page

TABLE A1—*Continued*

Temp (°C)	Salt (%)	SPP (%)	NaL (%)	Rep	No. of obs	RMSE	Time (min)[a]	CV of time (%)	*a*	*b*
65.0	2.50	0.30	1.5	1	7	0.545	3.43	52.73	11.313	2.967
65.0	2.50	0.30	1.5	2	7	0.378	9.06	67.42	10.849	1.869
65.0	2.50	0.30	0.0	1	6	0.464	2.43	32.63	11.485	3.916
65.0	2.50	0.30	0.0	2	6	0.516	1.05	55.96	14.847	2.466
65.0	2.50	0.15	2.5	1	7	0.404	1.94	48.61	13.422	2.322
65.0	2.50	0.15	2.5	2	7	0.297	20.34	76.71	10.344	1.535
65.0	2.50	0.00	2.5	1	6	0.253	1.33	34.05	14.350	2.152
65.0	2.50	0.00	2.5	2	6	0.137	13.70	46.27	12.037	1.119
65.0	1.50	0.30	2.5	1	5	0.179	2.47	26.79	12.970	2.207
65.0	1.50	0.30	2.5	2	8	0.227	12.01	51.76	11.450	1.415
65.0[b]	0.00	0.30	2.5	1	5	0.089	50.59	58.49	12.173	0.712
65.0	0.00	0.30	2.5	2	7	0.459	7.43	92.37	11.947	1.506
65.0	0.00	0.00	0.0	1	8	0.411	5.64	37.86	10.161	2.780
65.0	0.00	0.00	0.0	2	9	0.896	2.56	47.12	10.755	4.485
65.0	0.00	0.00	0.0	1	6	0.972	3.59	69.22	9.354	4.390
65.0	0.00	0.00	0.0	2	8	0.760	2.44	36.73	10.250	5.277
65.0	0.75	0.45	4.0	1	11	0.211	5.25	6.49	2.415	7.568
65.0	0.75	0.45	4.0	2	8	0.282	4.54	6.85	−1.850	11.123
65.0	3.75	0.45	1.0	1	13	0.356	7.39	6.94	−2.933	8.951
65.0	3.75	0.45	1.0	2	8	0.268	9.55	11.52	0.816	6.273
71.1	0.00	0.00	0.0	1	5	0.024	0.57	2.65	16.441	2.597
71.1	0.00	0.00	0.0	2	5	0.193	1.44	41.33	14.309	1.813
71.1	3.00	0.40	3.0	1	6	0.323	0.72	27.43	16.025	3.274
71.1	3.00	0.40	3.0	2	5	0.779	1.74	43.21	11.644	6.002
71.1	3.00	0.40	1.5	1	4	0.252	0.52	27.42	16.648	2.568
71.1	3.00	0.40	1.5	2	6	0.374	1.38	15.79	12.865	6.545
71.1	3.00	0.15	3.0	1	4	0.265	0.41	12.18	20.302	5.942
71.1	3.00	0.15	3.0	2	5	0.282	0.54	16.98	17.611	4.280
71.1	3.00	0.15	1.5	1	6	0.639	0.62	14.80	19.672	9.915
71.1	3.00	0.15	1.5	2	7	0.431	0.62	9.72	19.978	10.386
71.1	2.50	0.00	2.5	1	6	0.268	0.34	13.05	20.809	5.450
71.1	2.50	0.00	2.5	2	4	0.177	0.46	42.41	16.574	2.050
71.1	1.50	0.40	3.0	1	6	1.108	0.45	21.60	24.692	12.034
71.1	1.50	0.40	3.0	2	5	0.461	0.48	17.22	22.114	9.634
71.1	1.50	0.40	1.5	1	4	0.057	0.33	5.37	18.601	3.322
71.1	1.50	0.40	1.5	2	6	0.207	1.55	50.06	14.280	1.571
71.1	1.50	0.15	3.0	1	6	0.264	0.61	33.73	16.096	2.252
71.1	1.50	0.15	3.0	2	5	0.586	0.96	79.65	15.057	2.171
71.1	1.50	0.15	1.5	1	3	.	0.66	0.00	15.734	1.865
71.1	1.50	0.15	1.5	2	5	0.567	0.45	84.44	16.489	1.920
71.1	0.00	0.00	0.0	1	8	0.468	0.46	18.52	19.914	6.451
71.1	0.00	0.00	0.0	2	7	0.581	0.58	19.47	18.963	7.438

[a] Time to obtain 6.5-log lethality.
[b] Identified as outlier data.

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